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Comparative Study of the Susceptibilities of Major Epidemic Clones of Methicillin-Resistant *Staphylococcus aureus* to Oxacillin and to the New Broad-Spectrum Cephalosporin Ceftobiprole[▽]

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Multidrug-resistant strains of *Staphylococcus aureus* continue to increase in frequency worldwide, both in hospitals and in the community, raising serious problems for the chemotherapy of staphylococcal disease. Ceftobiprole (BPR; BAL9141), the active constituent of the prodrug ceftobiprole medocaril (BAL5788), is a new cephalosporin which was already shown to have powerful activity against a number of bacterial pathogens, including *S. aureus*. In an effort to test possible limits to the antibacterial spectrum and efficacy of BPR, we examined the susceptibilities of the relatively few pandemic methicillin-resistant *S. aureus* (MRSA) clones that are responsible for the great majority of cases of staphylococcal disease worldwide. We also included in the tests the highly oxacillin-resistant subpopulations that are present with low frequencies in the cultures of these clones. Such subpopulations may represent a natural reservoir from which MRSA strains with decreased susceptibility to BPR may emerge in the future. We also tested the efficacy of BPR against MRSA strains with reduced susceptibility to vancomycin and against MRSA strains carrying the enterococcal vancomycin resistance gene complex. BPR was shown to be uniformly effective against all these resistant MRSA strains, and the mechanism of superb antimicrobial activity correlated with the strikingly increased affinity of the cephalosporin against penicillin-binding protein 2A, the protein product of the antibiotic resistance determinant *mecA*.

Since the introduction of antibiotics in the 1940s, *Staphylococcus aureus*, a frequent cause of potentially life-threatening hospital-borne infections, has become increasingly resistant to most chemotherapeutic agents. In 1960, the first clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) appeared in the clinical environment and multidrug-resistant derivatives of MRSA clones began to spread worldwide, initially in the hospital environment but more recently in the community as well (11, 22). To treat MRSA infections, new antibiotics were developed, and beginning in the mid-1990s, the glycopeptide antibiotic vancomycin became the therapy of first choice for MRSA infections (3). Recently, MRSA isolates with reduced susceptibilities to vancomycin (vancomycin-intermediate *S. aureus* [VISA]) began to appear in several countries (4, 20, 25, 27) and MRSA strains that had acquired the enterococcal *vanA* gene complex (vancomycin-resistant *S. aureus* [VRSA]) were also recovered from clinical specimens. These VRSA strains also carried the β-lactam resistance gene *mecA* and were therefore resistant to both glycopeptide and β-lactam antibiotics (4, 24).

Ceftobiprole (BPR), the active component of the water-soluble prodrug ceftobiprole medocaril (BAL5788) (19), is a new cephalosporin that was shown to have powerful activity against many gram-positive pathogens (2, 15).

The primary purpose of this study was to determine the potency of this novel drug against highly oxacillin-resistant MRSA strains that exist at low frequencies in cultures of the most widely spread pandemic clones of MRSA (10). Under selective pressure, such subpopulations of highly β-lactam-resistant cells could become the source of lineages with increased BPR MICs. We also included in this study two MRSA strains that carried the enterococcal vancomycin resistance gene (VRSA strains) and two MRSA strains with reduced susceptibility to vancomycin (VISA strains).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. Mu50 was the first known MRSA isolate with reduced susceptibility to vancomycin (VISA) and was recovered from clinical specimens in Japan in 1996 (20). Another, more recent VISA isolate, isolate JH9, was recovered in the United States in 2000 from a bacteremic patient who underwent extensive chemotherapy with vancomycin during a 2-month period (25). VRS1 (HIP11714) is a highly VRSA clinical isolate that carries the enterococcal *vanA* gene complex and was obtained from the NARSA strain collection. Strain COLVA is a highly vancomycin-resistant transconjugant generated by introducing a plasmid-associated copy of Tn*1546* from donor isolate VRS1 into MRSA strain COL (24).

S. aureus strains were grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) with aeration at 37°C or on tryptic soy agar (TSA; Difco Laboratories) at 37°C. Bacterial growth was monitored by measurement of the optical density at 620 nm (OD_{620}) with a spectrophotometer (model 2800; UNICO, Dayton, NJ). VRSA isolates COLVA and VRS1 were grown overnight in 5 ml of TSB supplemented with 10 μg/ml of vancomycin in order to induce the expression of the *vanA* resistance gene. Overnight cultures of COLVA and VRS1 were then centrifuged at 3,700 rpm at 4°C for 10 min, and the resulting pellets were washed twice with 5 ml of TSB to remove the vancomycin and were resuspended in 5 ml of TSB before they were spread on TSA plates for determination of their population analysis profiles (PAPs).

Antibiotics. BPR (provided by Johnson & Johnson Pharmaceutical Research and Development, L.L.C., Raritan, NJ) was prepared by the addition of 99 μl of dimethyl sulfoxide and 10 μl of glacial acetic acid to 1.5 mg of powder, and the mixture was then diluted with 891 μl of distilled water. Vancomycin and oxacillin were purchased from Sigma, St. Louis, MO.

Cloning of the *mecA* gene, excluding the sequence encoding the membrane anchor. The *mecA* gene, which encodes the extracellular domain of penicillin-binding protein (PBP) 2A, was amplified by PCR from plasmid pSTSW-2C

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TABLE 1. *S. aureus* strains used in this study[a]

Strain	Isolation		Molecular typing				Oxacillin resistance phenotype
	Origin	Yr	Clonal type	MLST ST	SCC*mec* type	*spa* type	
E2125	Denmark	1964	Archaic	247	I	YHFGFMBQBLO	Hetero
HPV107	Portugal	1992	Iberian	247	IA	YHFGMBQBLO	Hetero
BK2464	United States	1996	NY/JP	5	II	TJMBDMGMK	Hetero
HDE288	Portugal	1996	Pediatric	5	VI	TJMBDMGMK	Hetero
BK2529	United States	1996	Clone V	8	IVd	YHGCMBQBLO	Hetero
HAR24	United Kingdom	1993	EMRSA16	36	II	WGKAKAOMQQQ	Hetero
HAR22	United Kingdom	1993	EMRSA15	22	IV	$TJEJNF_2MNF_2OMOKR$	Hetero
USA300	United States	NA	CA	8	IVa	YHGFMBQBLO	Hetero
COL	United Kingdom	1965	Archaic	250	I	YHFGFMBQBLO	Homo
HU25	Brazil	1993	Brazilian	239	IIIA	XKAOMQ	Homo
VRS1	United States	2002	NY/JP	5	II	TJMBDMGMK	Homo
COLVA	United States	2002	Archaic	250	I	YHFGFMBQBLO	Homo
Mu50	Japan	1996	NY/JP	5	II	TJMBDMGMK	Homo
JH9	United States	2000	NY/JP	105	II	TJMBDMGMK	Hetero

[a] Abbreviations: MLST, multilocus sequence typing; ST, sequence type; SCC*mec*, staphylococcal chromosomal cassette *mec*; NA, not available; CA, community associated; Homo, homogeneous; Hetero, heterogeneous.

carrying the *mecA* gene from *S. aureus* COL (30) with primers SPBP2AD (5′-ACTACCATGGCTTCAAAAGATAAAGAAATTAATA-3′) and SPBP2AR (5′-ATCTATCTCGAGTTATTCATCTATATCGTATT-3′). Recognition sequences of restriction endonucleases NcoI and XhoI (underlined in the sequences of primers SPBP2AD and SPBP2AR, respectively) were incorporated into the primers (17) and used for ligation of the PCR product at the corresponding sites in expression vector pET24d(+). The recombinant plasmid was introduced into *Escherichia coli* DH5α, and the correct sequence of the *mecA* gene was confirmed by nucleotide sequencing. The recombinant plasmid was then used to transform *E. coli* Tuner(DE3) competent cells (Novagen).

Purification of soluble PBP 2A. PBP 2A was purified by the use of three steps of chromatography. Four milliliters of an overnight culture were inoculated into 400 ml of fresh LB medium containing 30 μg/ml of kanamycin. Cells were grown at 37°C with shaking at 200 rpm until the OD_{600} reached 0.6, and then protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.4 mM. The cells were further incubated for 16 h at 25°C. Bacteria were collected by centrifugation at 5,000 × *g* for 20 min at 4°C and washed with 20 mM Tris-HCl, pH 7.6 (buffer A). The following purification steps were performed at 4°C. Cells were suspended in 30 ml of buffer A containing lysozyme (200 μg/ml), DNase I (20 μg/ml), RNase A (20 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) and incubated on ice for 30 min, followed by cell disruption for 10 min with a W-225 sonicator (Heat System-Ultrasonics, Inc.) with the following settings: pulsed, 40% duty cycle, and 4 output control. Unbroken cells were removed by centrifugation at 14,000 × *g* for 50 min.

The supernatant was applied onto a DEAE-anion exchange column (2.5 by 30 cm; Bio-Rad) equilibrated in buffer A. Proteins were eluted at a flow rate of 4 ml/min with a linear gradient from 0 to 1 M NaCl in buffer A. PBP 2A was eluted at 0.1 to 0.2 M NaCl, and the presence of PBP 2A in the fractions was confirmed by a Bocillin FL-binding assay (31). The fractions containing PBP 2A were collected, concentrated, and dialyzed against 2 liters of 50 mM morpholinepropane sulfonic acid, pH 7.0 (buffer B). The protein solution was loaded onto a High S cation-exchange column (2.5 by 30 cm; Bio-Rad) equilibrated in buffer B. Proteins were eluted at a flow rate of 5 ml/min with a linear gradient from 0 to 1 M NaCl in buffer B. PBP 2A was collected from the 0.5 to 0.6 M NaCl fractions, and its presence in the fractions was also checked by the Bocillin FL-binding assay. The fractions were combined, concentrated, and dialyzed against 2 liters of 50 mM sodium phosphate, pH 7.2, and 150 mM NaCl (buffer C). The concentrated proteins were loaded onto a Sephacryl S-300 size-exclusion column (1.5 by 100 cm; Amersham Biosciences). PBP 2A was eluted with buffer C at a flow rate of 0.3 ml/min. A total of 15 mg of PBP 2A was obtained after the final chromatographic step with about 97% purity, as checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The protein was concentrated to 5 mg/ml, and the concentration was determined with a bicinchoninic acid protein assay kit (Pierce).

Antibiotic susceptibility testing. The antibiotic resistance levels of the bacterial strains and the synergistic activities of the antibiotics tested were determined by Etest by following the recommendations of the manufacturer (AB Biodisk, Solna, Sweden) and by the PAP method, as described previously (29), on agar plates containing increasing concentrations of oxacillin, BPR, or vancomycin. The numbers of CFU were counted after incubation at 37°C for 48 h.

Selection of MRSA subpopulations with high-level resistance to oxacillin and/or BPR. Cultures of most MRSA strains are heterogeneous: in addition to the majority of cells, which exhibit a low level of antibiotic resistance, cultures of such strains also contain subpopulations of highly resistant bacteria which are present at various (low) frequencies in the cultures. We have been referring to these highly resistant subpopulations as "homo*" (10). It was shown that under the appropriate conditions, these highly resistant homo* colonies can be selected to produce homogeneous cultures of highly resistant bacteria.

Mutants that were able to grow on agar plates containing high concentrations of either oxacillin or BPR were picked from the agar plates and restreaked on antibiotic-free TSA in three consecutive passages in order to test the stability of the phenotype. Such homogeneous homo* cultures were then tested for their susceptibilities to oxacillin and/or BPR by the population analysis method.

Time-kill curve studies. The rate of antibiotic-induced loss of viability was measured in exponentially growing cultures of COL that at zero time (corresponding to an OD_{620} of 0.2, or about 10^8 CFU per ml) received concentrations of BPR or oxacillin at 10 times the corresponding MIC. Control cultures received no antibiotics. Aliquots of the cultures were removed at various time intervals, serially diluted, and plated on TSA. The colonies were counted after 48 h of incubation at 37°C.

Membrane purification and penicillin-binding assays. Membranes were prepared from cells grown to late exponential phase, as described previously (26). The preparations (150 μg of proteins per sample) were incubated with increasing concentrations of oxacillin or BPR for 10 min at 30°C and then labeled with 20 μg/ml of benzyl[^{14}C]penicillin potassium salt (GE Healthcare) for 10 min at 37°C. Addition of an excess of unlabeled benzylpenicillin (1,000 μg/ml in 10% [wt/vol] SDS) was used to stop the reaction, and samples were processed as described previously (26).

Determination of BPR and oxacillin IC_{50}s for purified PBP 2A. The relative binding affinities of purified PBP 2A for BPR and oxacillin were compared in a competition assay with Bocillin FL as a reagent (31), and the results were compared to the results of the penicillin-binding assays performed with membrane fractions. Purified PBP 2A (0.8 μM) was incubated with either BPR or oxacillin at concentrations ranging from 0.5 μM to 20 μM for 30 min at 30°C in a final volume of 30 μl, followed by addition of 20 μM of Bocillin FL (Invitrogen) and additional incubation for 10 min at 37°C. The reaction was quenched by adding 15 μl of 3× SDS-PAGE sample buffer and boiling for 3 min at 100°C. The reaction mixtures were loaded on a 10% SDS-polyacrylamide gel, and the gel was then scanned with a Typhoon 9400 scanner (excitation at 488 nm; GE Healthcare) to visualize the Bocillin FL-labeled PBP 2A. The intensity of fluorescence was quantified by the use of ImageQuant software (Molecular Dynamics).

The 50% inhibitory concentrations (IC_{50}s) of BPR and oxacillin for purified PBP 2A were evaluated by use of a Bocillin FL-binding assay as described above with either BPR at concentrations ranging from 0.5 to 200 μM or oxacillin at concentrations ranging from 5 to 6,000 μM. The IC_{50}s were calculated by fitting

TABLE 2. Susceptibilities to oxacillin and BPR of major epidemic MRSA clones and their oxacillin- and BPR-resistant subpopulations

Strain	MIC (μg/ml) for cell majority		Subpopulation growing on (μg/ml):		MIC (μg/ml) for OXA-resistant subpopulation		BPR MIC (μg/ml) for BPR-resistant subpopulation
	OXA[a]	BPR	OXA	BPR	OXA	BPR	
E2125	6	0.8	400	6	400	0.8	1.5
HPV107	0.8	0.8	400	3	100	0.8	0.8
BK2464	12.5	1.5	400	1.5	200	1.5	3
HDE288	0.8	0.8	400	3	200	1.5	1.5
BK2529	1.5	1.5	400	3	200	1.5	1.5
HAR24	100	1.5	800	3	800	1.5	3
HAR22	100	0.8	400	1.5	800	0.8	3
USA300	0.8	1.5	800	1.5	400	1.5	0.8
COL	>800	3	>800	3	ND[b]	ND	3
HU25	400	3	800	3	ND	ND	3

[a] OXA, oxacillin.
[b] ND, not determined.

the data to GraFit (version 4.0) software (Erithacus Software Ltd., United Kingdom).

Conformational change of PBP 2A by BPR and oxacillin. PBP 2A (1 μM) was dissolved in 20 mM sodium phosphate buffer, pH 7.2, containing 30 mM NaCl in order to determine changes induced in the circular dichroic (CD) spectra by the addition of 20 μM of BPR or oxacillin. The CD spectra were recorded in an AVIV-62 CD spectrometer (1-cm path length; AVIV Biomedical, Inc.). PBP 2A was incubated in the absence and in the presence of β-lactam antibiotics for 30 min at 25°C before the CD spectra were recorded. The effect of each β-lactam by itself on the CD spectra, in the absence of PBP 2A, was subtracted (16, 17). BPR dissolved in 10% acetonitrile and 1% phosphoric acid solution at 1 mM was used as a stock solution for the CD experiments.

Peptidoglycan purification and analysis by high-pressure liquid chromatography (HPLC). Peptidoglycan was prepared from *S. aureus* cells by methods described previously (8).

RESULTS AND DISCUSSION

Recent studies in several laboratories have already documented the powerful antimicrobial activity of the newly developed β-lactam antibiotic BPR against several species of bacteria (13). While the greatly improved MIC of BPR is welcome news, experience with previously developed antimicrobial agents shows that the appearance of BPR-resistant *S. aureus* strains in the clinical environment is only a matter of time. It was also documented that strains resistant to a new antimicrobial agent often appeared from bacterial lineages that have already become resistant to previously extensively used antimicrobials, thus generating multidrug-resistant strains (12). Thus, newly emerging MRSA isolates were often also resistant to penicillin and other previously used therapeutic agents, such as streptomycin, tetracycline, and erythromycin (6). This also seems to be the case with the most recently emerging VISA and VRSA strains, which invariably carry the mechanism of methicillin resistance, reflecting the primary therapeutic use of these agents against infections caused by multidrug-resistant MRSA strains. In view of this background of the history of antimicrobial resistance, we performed experiments to identify a potential source of resistant strains that may emerge upon the introduction of BPR into clinical use.

A most likely source for such BPR-resistant strains appears to be the highly oxacillin-resistant subpopulations that are present at various low frequencies in cultures of most MRSA lineages with a heterogeneous phenotype (10). In the studies described here, we examined the activity of BPR against such highly oxacillin-resistant subpopulations of staphylococci which are present in the relatively few major pandemic MRSA clones that are responsible for most MRSA disease worldwide both in hospitals and in the community (1).

Efficacy of BPR against major epidemic MRSA clones. Table 1 shows the geographic origin, date of isolation, clonal type, and genetic backgrounds (sequence type, staphylococcal chromosomal cassette *mec* type, *spa* type), as well as the oxacillin resistance phenotypes of several pandemic MRSA lineages. The MICs for oxacillin and BPR exhibited by both the majority and the minority populations of bacteria present in the cultures of the heteroresistant strains are shown in Table 2.

The PAPs of these bacterial strains were determined for both oxacillin and BPR (Fig. 1). The most interesting features of these PAPs are the sharply contrasting shapes of the population analysis curves obtained with the two antibiotics when the same bacterial strains are compared. In contrast to the oxacillin-heteroresistant phenotypes of most MRSA clones, the shapes of the population analysis curves obtained with BPR indicates a virtual absence or a greatly reduced frequency of the highly resistant subpopulations (9). Indeed, the distribution of BPR MICs against the majority of cells and the resistant minority population appears to be in the same range of 0.8 to 6 μg/ml (Table 2).

In order to examine this issue more closely, we picked single colonies of bacteria that grew on the agar plates containing 100 μg/ml or 200 μg/ml of oxacillin (Fig. 1). After establishing the stability of high-level resistance in these colonies through serial passages, we used them as inocula for liquid cultures, which were then reexamined for their profiles of susceptibility to oxacillin and BPR. The stability of the resistant phenotype in most of these subpopulations has been demonstrated before (29). Figure 1 shows that these original "minority" subpopulations produced virtually homogeneous oxacillin-resistant cultures of MRSA with high MICs (Table 2). We also examined the BPR susceptibilities of these highly oxacillin-resistant cultures. In striking contrast to the nearly homogeneous and high oxacillin MICs, the MIC for BPR remained uniformly low for each of the strains tested (Fig. 1 and Table 2). In fact, the spread of BPR MICs against these highly oxacillin-resistant subpopulations was within the same narrow range of 0.8 to 1.5 μg/ml already demonstrated against both the majority and the

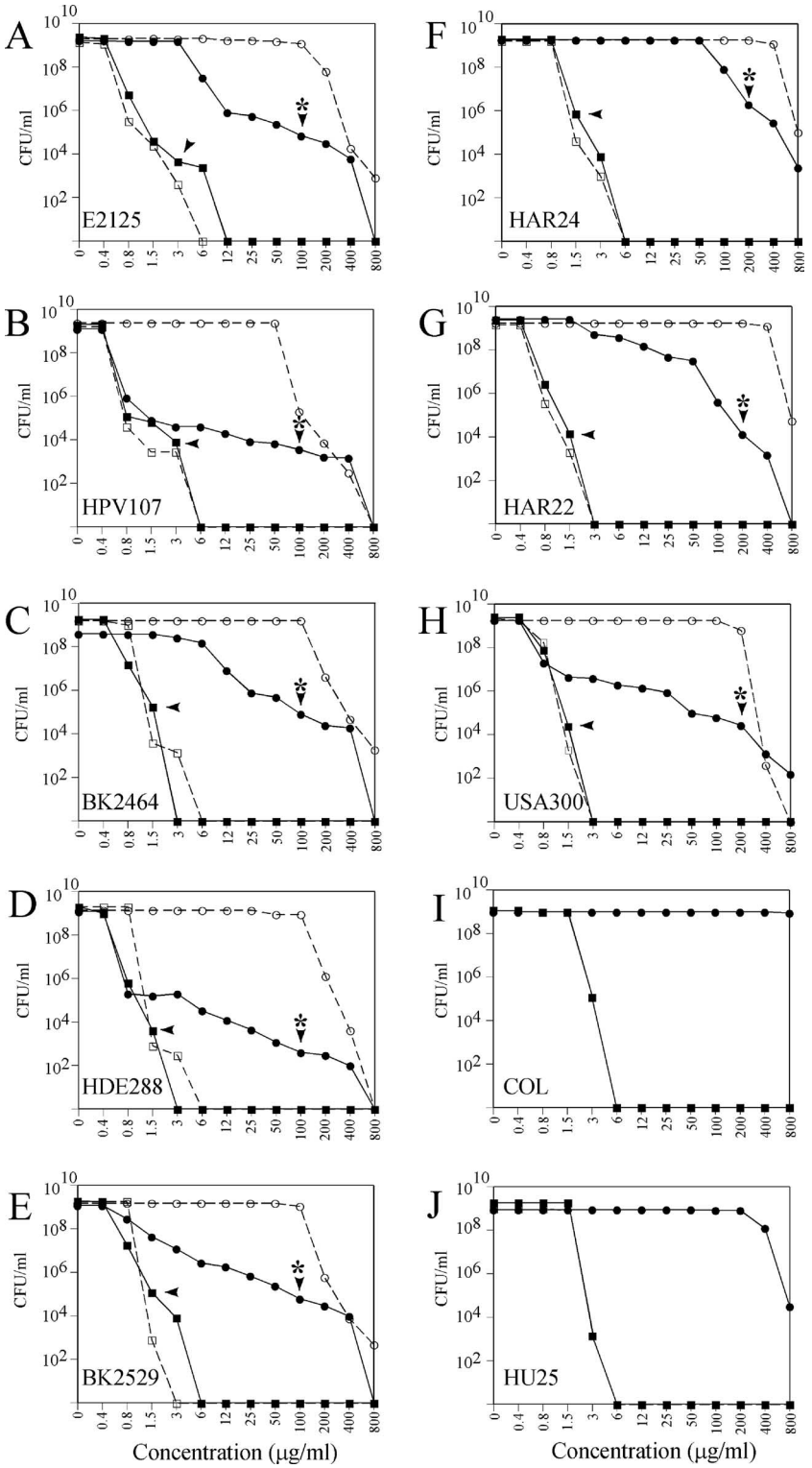

FIG. 1. Oxacillin and BPR susceptibility profiles of major epidemic MRSA clones and their homogeneous oxacillin-resistant subpopulations. Aliquots of an overnight culture were plated after serial dilution on TSA containing increasing concentrations of oxacillin (circles) or BPR (squares). The numbers of CFU were counted after incubation for 48 h at 37°C. The antibiotic susceptibility profiles (closed symbols and solid lines) were first determined for E2125 (A), HPV107 (B), BK2464 (C), HDE288 (D), BK2529 (E), HAR24 (F), HAR22 (G), USA300 (H), COL (I), and HU25 (J). Colonies were then picked from the subpopulations that were able to grow in the presence of high concentrations of oxacillin (indicated by the arrowheads with asterisks), and the oxacillin and BPR susceptibility profiles were further determined (open symbols and dashed lines). Colonies were also picked from the subpopulations that were able to grow in the presence of elevated concentrations of BPR (arrowheads), and the BPR susceptibility profiles of the bacteria were determined (see Table 2).

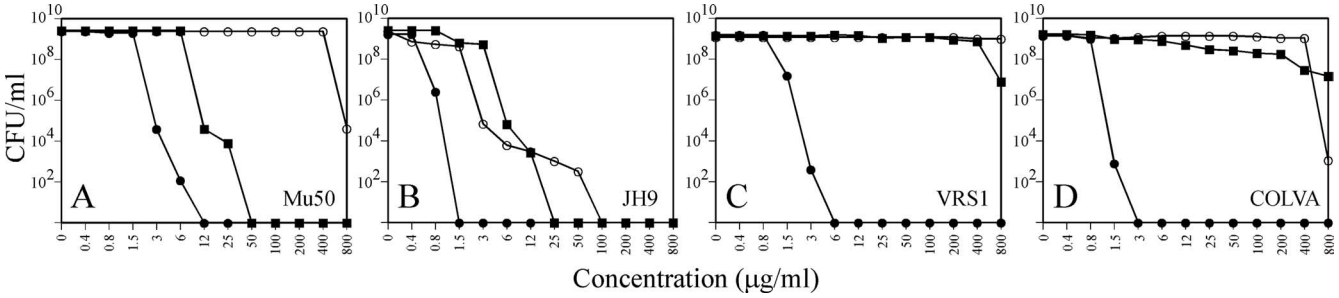

FIG. 2. Vancomycin, oxacillin, and BPR susceptibility profiles of VISA and VRSA strains. Aliquots of overnight cultures were plated on TSA containing increasing concentrations of vancomycin (closed squares), oxacillin (open circles), or BPR (closed circles). The numbers of CFU were counted after incubation at 37°C for 48 h. The antibiotic susceptibility profiles were determined for VISA strains Mu50 (A) and JH9 (B) and for VRSA strains VRS1 (C) and COLVA (D).

minority populations of the original MRSA clones (Table 2). The selection of highly oxacillin-resistant subpopulations from individual MRSA strains did not increase the BPR MICs.

We also picked single colonies of bacteria that grew on agar plates containing 1.5 or 3 μg/ml of BPR (Fig. 1) and determined their susceptibilities to BPR. The BPR MICs for the BPR-resistant subpopulations remained unchanged (Table 2).

Activity of BPR against MRSA strains with decreased susceptibility to vancomycin (VISA). We tested the BPR susceptibilities of two clinical VISA isolates with somewhat different mechanistic features. Strain Mu50 represents the first VISA isolate recovered from clinical specimens in Japan in 1996 (20). The second VISA strain, strain JH9, was recovered from a bacteremic patient in the United States in 2000 (25). While both of these VISA strains have vancomycin MICs within a similar range (6 to 12 μg/ml), they differed in their autolytic phenotypes (18, 28). Both Mu50 and JH9 are single-locus variants of NY/JP clone ST5 (sequence type 5) and show homogeneous (Mu50) and heterogeneous (JH9) resistance to oxacillin. While the oxacillin MICs for the majority of cells for Mu50 and JH9 were >800 and 3 μg/ml respectively, the corresponding MICs for BPR were 3 and 0.8 μg/ml (Fig. 2A and B).

Activity of BPR against highly VRSA strains carrying the enterococcal Tn*1546* vancomycin resistance gene complex. Clinical VRSA isolate VRS1 showed a high level of and homogeneous resistance to both oxacillin and vancomycin (MICs, >800 μg/ml) (Fig. 2C). A second VRSA strain, named COLVA, was generated in the laboratory by introducing a plasmid carrying the Tn*1546* transposon from clinical strain VRS1 into highly oxacillin-resistant MRSA strain COL (24). Both VRS1 (Fig. 2C) and COLVA (Fig. 2D) shared a very low MIC for BPR of 1.5 μg/ml. This is in contrast to the uniformly high MICs of over 800 μg/ml for both oxacillin and vancomycin.

Earlier studies (24) have demonstrated that oxacillin at sub-MICs can effectively lower the vancomycin MIC of strain COLVA and convert the homogeneous vancomycin-resistant phenotype of this strain to a heterogeneous one. We tested the synergistic effect of BPR on the vancomycin resistance phenotype of VRSA strains VRS1 and COLVA. Addition of BPR at a concentration of 1 μg/ml to the vancomycin-containing agar plates produced cultures with the heterogeneous vancomycin susceptibility phenotype in both strain VRS1 (Fig. 3A) and strain COLVA (Fig. 3B), similar to what was already shown for oxacillin in the case of strain COLVA (24), and resulted in a drastic reduction of the vancomycin MIC (Fig. 4). The big difference between the two synergists was in the absolute concentrations of the β-lactam compounds necessary to substantially reduce the level of vancomycin resistance in the majority of cells. While oxacillin concentrations as high as 50 μg/ml

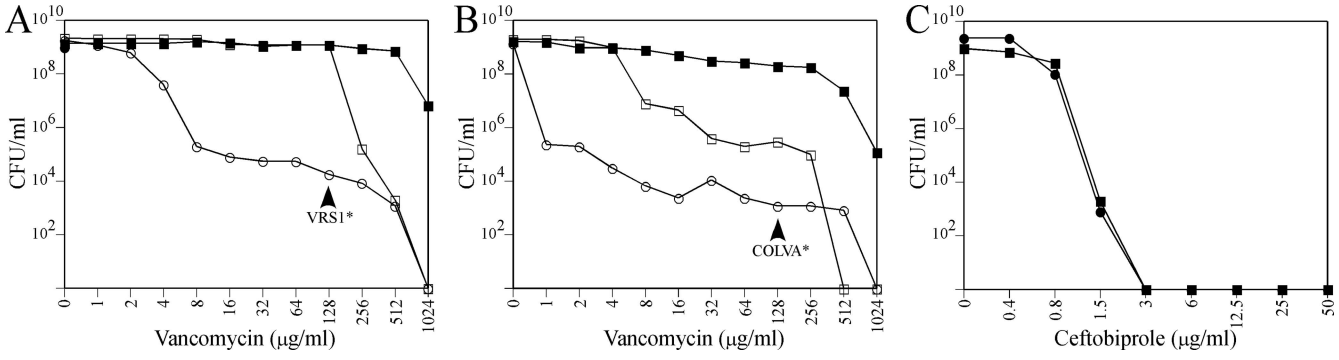

FIG. 3. Inhibitory effects of BPR on the expression of the vancomycin resistance phenotype in VRSA strains VRS1 and COLVA. Aliquots of overnight cultures of strains VRS1 (A) and COLVA (B) were plated on TSA containing increasing concentrations of vancomycin in the absence of BPR (closed squares) or in the presence of a constant concentration (1 μg/ml) of BPR (open circles). Colonies were picked from the subpopulations of VRS1 or COLVA growing on plates containing 128 μg/ml of vancomycin and 1 μg/ml BPR (as indicated by arrows). Cultures of these colonies, named VRS1* and COLVA*, respectively, were tested for their vancomycin susceptibility profiles in the presence of a constant concentration (1 μg/ml) of BPR (open squares). (C) BPR susceptibility profiles of VRS1* (closed squares) and COLVA* (closed circles).

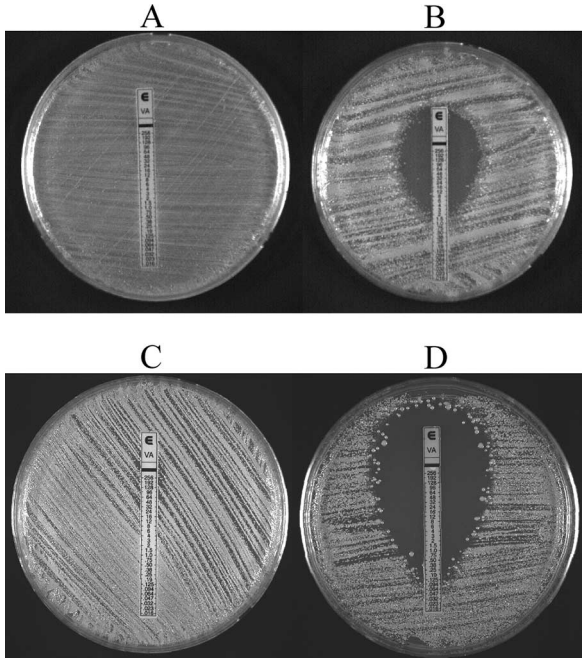

FIG. 4. Synergistic effect of BPR on the vancomycin resistance of VRSA strains COLVA and VRS1. Strains VRS1 (A and B) and COLVA (C and D) were spread on TSA plates without (A and C) or with (B and D) 1 μg/ml of BPR added to the agar medium. The vancomycin susceptibilities were evaluated after overnight incubation at 37°C by the vancomycin Etest method.

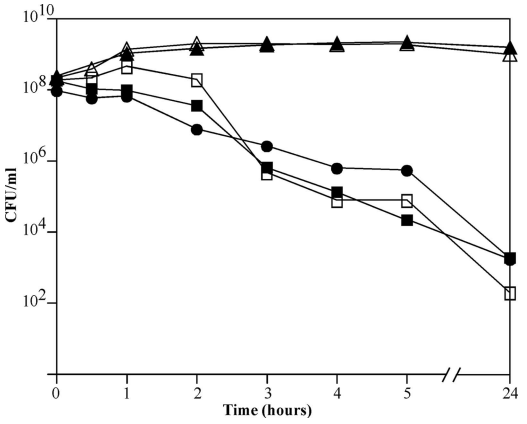

FIG. 5. Susceptibility of MRSA strain COL to the bactericidal effects of oxacillin and BPR. The viability of MRSA strain COL was determined during exposure to 10× the MIC of oxacillin (closed circles) or BPR (closed squares) and was compared to that of a culture grown in antibiotic-free medium (closed triangles). Exponentially growing cultures were exposed to the antibiotic at an OD_{620} of 0.2 (zero time), and aliquots were removed at various intervals to determine the viable counts. Bacterial cells recovered from the first exposure to BPR for 24 h were again inoculated into fresh medium in the absence of BPR (empty triangles) or in the presence of the same concentration of BPR (empty squares), and the viable counts were determined.

were necessary to reduce the level of vancomycin resistance (and produce the heterogeneous phenotype) (24), a drastic reduction of the vancomycin MIC of VRS1 and COLVA was achieved with as little as 1 μg/ml of BPR (Fig. 4).

Similar to the case of heterogeneous oxacillin-resistant strains, we selected from the synergy experiments highly vancomycin-resistant subpopulations (Fig. 3A and B) of VRS1 and COLVA, named VRS1* and COLVA*, respectively, that produce cultures composed of these more highly vancomycin-resistant subpopulations. We tested the susceptibility to BPR of VRS1* and COLVA*. Figure 3C shows that such highly vancomycin-resistant subpopulations (VRS1* and COLVA*) retained, unaltered, extremely low BPR MICs (1.5 μg/ml).

Comparison of bactericidal and bacteriolytic activities of BPR and oxacillin. The susceptibility of MRSA strain COL to the killing and lytic effects of oxacillin and BPR were compared by exposing exponentially growing cultures of the bacteria at mid-log phase of growth to concentrations of the two antibiotics equivalent to 10× the MICs. The cultures were incubated with the two antibiotics under aerobic conditions, and at different time intervals the number of surviving bacteria was determined. Figure 5 shows that oxacillin and BPR, used at equivalent concentrations in terms of multiples of their MICs, had indistinguishable bactericidal activities. After 24 h of incubation with 10× the MICs of the antibiotics, both oxacillin and BPR reduced the initial viable titer of the bacteria from 10^8 to about 10^3 CFU/ml.

Bacterial cells surviving the 24-h exposure to 10× the MIC of BPR were diluted to remove residual antibiotics and used to generate fresh cultures, which were again exposed to the same 10× MIC of BPR. Such a second exposure to BPR did not enrich the culture in bacterial cells that would have become less susceptible to the bactericidal activity of the antibiotic (Fig. 5).

In parallel experiments of exactly the same design used to generate the results shown in Fig. 5, the bacteriolytic activities of BPR and oxacillin were compared by monitoring the decrease in OD_{620} against time. No differences in the lytic potentials of BPR and oxacillin could be detected (data not shown).

Mechanism of action of BPR. The remarkable antimicrobial activity of BPR against MRSA strains, including the highly oxacillin-resistant subpopulations produced by each one of the widely spread epidemic MRSA clones, raises the possibility that BPR may have a novel, as yet not fully characterized property not shared by other β-lactam antibiotics, and such a hypothetical mechanism may be the basis of BPR's superior antimicrobial power.

In order to test this, we performed several assays (assays 1 through 4) to probe the mode of action of BPR in *S. aureus*.

(i) Assay 1: titration of affinity of BPR for PBPs of MRSA strain COL. Membrane preparations from a PBP 2 insertional mutant of MRSA strain COL (mutant RU130) were used to determine the relative affinities of oxacillin and BPR against the PBPs of the bacteria by an in vitro competition assay with radioactive benzylpenicillin. Membrane preparations were preincubated with increasing concentrations of the various antibiotics, after which the preparations received a single common concentration of the radioactive penicillin reagent, as described in Materials and Methods. The rationale for using the PBP 2 insertional mutant was to allow the clear detection of PBP 2A free of PBP 2 in the penicillin-binding assay since these two PBPs have virtually identical migrations in SDS-

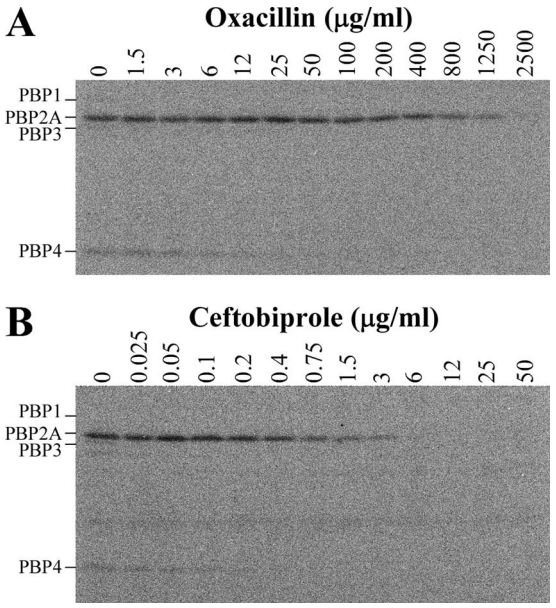

FIG. 6. Comparison of affinities of oxacillin and BPR for PBPs of *S. aureus* strain RU130. Membrane preparations (150 μg of proteins) were first incubated with increasing concentrations of oxacillin (A) or BPR (B) and then with a single saturating concentration of benzyl[^{14}C]penicillin. After SDS-PAGE, the gel was exposed to a tritium storage phosphor screen for 2 weeks.

polyacrylamide gels. Mutant RU130, in which Tn*551* was inserted into the transpeptidase domain of the protein, was shown to produce a truncated PBP 2 of faster mobility, thus enabling one to evaluate the affinity of PBP 2A free from interference (23).

Figure 6 shows the results of the PBP competition assays. While a concentration of oxacillin as high as 2,500 μg/ml was necessary to fully saturate PBP 2A, a BPR concentration as low as 6.0 μg/ml was sufficient to achieve this. The penicillin-binding assay with BPR also showed that, in parallel with its high affinity for PBP 2A, this β-lactam was also able to saturate PBPs 1, 3, and 4 as well within an extremely low range of concentrations.

(ii) Assay 2: IC_{50}s of BPR and oxacillin for purified PBP 2A. The Bocillin FL-binding assay with purified PBP 2A was carried out to confirm the penicillin-binding assay with membrane preparations described above. PBP 2A was incubated with different concentrations of BPR or oxacillin prior to addition of a constant concentration of Bocillin FL. As shown in Fig. 7, 66% of PBP 2A was inhibited by BPR at 10 μM, whereas only 5% of PBP 2A was inhibited by oxacillin used at the same 10 μM concentration. These data indicate that BPR has very high binding affinity for the purified PBP 2A protein compared to oxacillin. This is consistent with the result of the affinity titration assay with membrane preparations.

The IC_{50}s of these antibiotics for purified PBP 2A were 3.2 ± 0.4 μM (1.7 ± 0.2 μg/ml) for BPR and 710 ± 80 μM (320 ± 35 μg/ml) for oxacillin, indicating about a 220-fold higher efficiency of BPR than of oxacillin for the inhibition of PBP 2A. This IC_{50} of oxacillin for PBP 2A (320 ± 35 μg/ml) isolated from MRSA strain COL is similar to those (both 300 μg/ml) obtained for PBP 2A isolated from the MRSA strains 67-O and

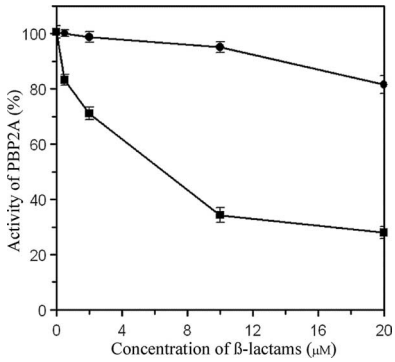

FIG. 7. Comparison of the affinities of BPR and oxacillin for purified PBP 2A. Purified PBP 2A was initially incubated with different concentrations (from 0.5 μM to 20 μM) of BPR (squares) or oxacillin (circles), and then Bocillin FL (20 μM) was added as a reporter substrate. After SDS-PAGE, the gel was immediately scanned with a fluoroimager (Typhoon 9400).

27R (5). The IC_{50} of BPR for PBP 2A (1.7 ± 0.2 μg/ml) is also comparable to those (~0.5 μg/ml and 0.9 μg/ml, respectively) reported for PBP 2A purified from MRSA strains P8-Hom (15) and OC 3726 (7). These IC_{50}s are remarkably low compared to the values obtained for other β-lactam antibiotics. The new cephalosporin BPR appears to exhibit the highest affinity of binding to PBP 2A among the β-lactam antibiotics studied so far (5, 7, 15).

(iii) Assay 3: conformational change of PBP 2A by BPR and oxacillin. The conformational change of PBP 2A by oxacillin was demonstrated by Fuda et al. (17). The conformational change of PBP 2A by BPR was investigated to elucidate if the binding of BPR to PBP 2A would occur in a way similar to that for oxacillin. Figure 8 shows the conformational change of PBP 2A either by BPR or by oxacillin. As mentioned before, since the binding affinity of oxacillin for PBP 2A is very low, a large conformational change of PBP 2A did not occur when the protein was exposed to 20 μM of oxacillin. However, the same concentration of BPR caused dramatic α-helical relaxation at 208 nm and 222 nm, as shown in Fig. 8. This result indicates not

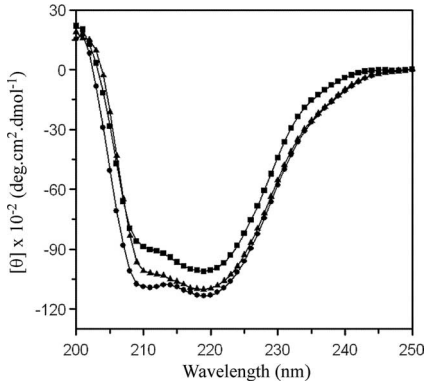

FIG. 8. Effects of β-lactam antibiotics on the circular dichroic spectra of PBP 2A. The conformational change of PBP 2A was determined by measuring the CD spectra in the absence of drug (circles) and in the presence of either BPR (squares) or oxacillin (triangles). Purified PBP 2A was incubated with β-lactam antibiotics for 30 min at 25°C, and then the CD spectra were recorded from 250 nm to 200 nm.

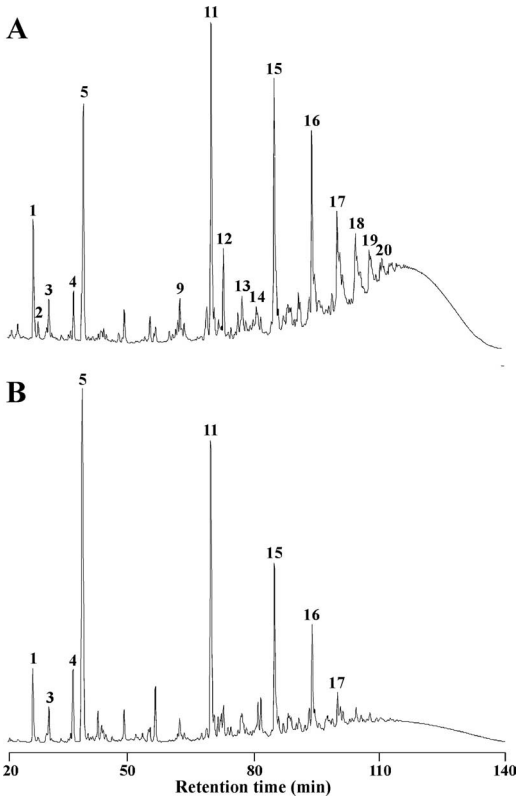

FIG. 9. HPLC elution profiles of peptidoglycan purified from MRSA strain COL grown in the absence and in the presence of BPR. Peptidoglycans were purified from 1-liter cultures grown at 37°C to an OD_{620} of 0.4 in the absence of BPR (A) or in the presence of a subinhibitory concentration (0.4 μg/ml) of BPR (B). Following digestion with mutanolysin, muropeptides were separated by HPLC and detected by measurement of the absorbance at 206 nm.

only that the mechanism of binding of BPR to PBP 2A may be similar to that of oxacillin but also that BPR may bind to the active site of the protein.

(iv) Assay 4: effects of BPR on composition and cross-linking of *S. aureus* cell wall. MRSA strain COL was grown with and without sub-MICs (0.4 μg/ml, or one-fourth of the MIC) of BPR, and the muropeptide composition of peptidoglycan was compared after separation on a reverse-phase HPLC column. Figure 9 compares the control and BPR-treated cultures. Bacteria grown in the presence of sub-MICs of BPR produced an HPLC profile characteristic of that for β-lactam-treated staphylococci: there was a drastic reduction in the contribution of highly cross-linked oligomers eluting from the column with retention times longer than 100 min. There was also a parallel increase in the proportion of monomers and less extensively cross-linked oligomers. This shift in cell wall composition is consistent with the activity of a transpeptidase inhibitor measured directly in terms of its effect on the cell wall composition.

The results of experiments 1 through 4 confirm that BPR is a highly effective transpeptidase inhibitor and that the mechanism of antimicrobial action of BPR against MRSA is related to its extremely high affinity for PBP 2A.

In view of recent reports of "superbugs," such as community-acquired MRSA reported in the public media (14), in which treatment with vancomycin failed, new antibiotics with high degrees of efficacy against such multidrug-resistant strains are urgently needed. Thus, the apparent success of the new cephalosporin BPR is extremely encouraging, since it reopens the possibility that antimicrobial agents effective against staphylococci which carry the *mecA*-dependent wide-spectrum resistance mechanism can be produced.

The exact enzymatic mechanism by which bacterial transpeptidases and transglycosylases (PBPs) recognize their in vivo muropeptide substrates has remained unclear, despite extensive efforts (21). This was particularly the case for PBP 2A, the active site of which appears to be accessible only to β-lactam inhibitors capable of inducing an initial allosteric type interaction which "opens up" the structure of this protein, as Fuda and colleagues recently demonstrated (16). The appearance of a cephalosporin such as BPR with powerful activity against MRSA strains should be encouraging for medicinal chemists, since it suggests that modification of the β-lactam core structure may still be a rewarding avenue for producing weapons effective against the sophisticated mechanism of resistance that has evolved in methicillin-resistant staphylococci (21).

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